I INTRODUCTION

Fungi

Fungi, are a kingdom of heterotrophic, achlorophyllous, single-celled, multinucleated, or multicellular organisms, including yeasts, molds, and mushrooms (Sharma, 1990). The branch of biology that deals with fungi is known as Mycology. It is derived from a Greek word Mykes, which means mushroom, and fungus is derived from a Latin word, which also means mushroom. Previously classified in the plant kingdom, fungi are non motile, like plants, but lack the vascular tissues (phloem and xylem). These organisms are transferred to a separate kingdom in Whittaker's Five kingdom classification in 1969, based on cell morphology and subsequently substantiated by molecular studies. Most fungi are capable of asexual (by fragmentation or spore formation) and sexual reproduction (produce gametes in specialized areas of the hyphae called gametangia). The gametes may be released to fuse into spores elsewhere, or the gametangia themselves may fuse. In some cases dikaryons [di = two, karyo = nucleus], which are found only among fungi, result when unspecialized hyphae fuse but their nuclei remain distinct for part of the life cycle.
Unlike algae or plants, fungi lack the chlorophyll necessary for photosynthesis and must therefore they exist as parasites, symbionts, or saprobes (http://www.infoplease.com/ce6/sci/A0819891.html). Typically they release digestive enzymes onto a living food source (parasitic types) or nonliving food source (saprophytic), partially dissolving it to make the necessary organic or inorganic nutrients while others are involved in symbiotic relationships, for example, lichens (a combination of a fungus and an alga or a cyanobacterium) and the mycorrhizae (symbiosis between a fungus and the roots of a vascular plant). Fungi are also used for beneficial purposes like food (30sps out of 10,000sps of mushroom), lipid production (Candida and Fusarium), bread-making, brewing, single cell proteins (Saccharomyces cervisiae), cheese production, antibiotic production (Penicillium spp), biofertilizers (mycorrhizae), herbicides (Gliocladium), insecticides, fungicides (Trichoderma), enzymes production (α-amylase from Aspergillus oryzae), plant growth hormones (Gibberellin from Gibberella fugikuroi) and used for tincturation (Usnea and Torula) (Vaidya, 1995). Some molds, in particular, release toxic chemicals (mycotoxins) that can result in poisoning or death. Various fungi can also cause serious damage to fruit harvests and other crops. Some fungi are pathogenic to humans and other animals. Such diseases are called mycoses or fungal infections.

Fungi causing diseases in animals fall into three categories: (Vaidya, 1995)

(i) Systemic infections, caused deep within the tissues (involving vital organs or nervous system) and are often fatal.

(ii) Superficial infections, caused mild on skin, nail or subcutaneous tissues (dermatophytes)

(iii) Intermediate infections, which will have severity between the two extremes.
It has been estimated by Hawksworth that only 5 percentage of the total fungal species in the world have been identified which constitute about 69,000 species out of an estimated 1,500,000 but hardly 300 are usually recognized as primary human pathogens, of which 10-15 are commonly encountered in routine clinical practice.

**Medical mycology**

Medical Mycology is the study of fungal epidemiology, ecology, pathogenesis, diagnosis and treatment in human beings. In 1835 **Augustino Bassi** in Italy established that a fungus, *Beauveria bassiana*, was the cause of disease in silk worms (*Bombyx mori*) called muscardine. On the basis of this finding he predicted that fungi can also cause infection in man. Medical Mycology attained recognition in the World sciences in 1910 when French dermatologist **Raymond Jacques Sabouraud** (1864-1936) published his monumental work on dermatophytes, “Les Teignes”. He has rightly been considered as father of Medical Mycology.

Although fungi were recognized much earlier, they were overshadowed by bacteriology and even by virology. Medical Mycology was considered like a “step child” and was the least bothered subject in the medical institutions as compared to other branches of medical science. The fungal infection has such an impact that nowadays no medical personnel can ignore mycology whatever may be his speciality. This “obligatory” attention is due to the fact that non-pathogenic forms cause serious secondary infections leading to death in immuno-compromised patients and these forms are turning towards pathogenic or opportunistic causing infections in healthy individuals and set a challenge to clinicians. The field of Medical Mycology, which was restricted to case reports in the 19th century, has attained a status of full-fledged subject, an important branch of medical science by the end of 20th century.
As the fungal infections are not notifiable as that of viral, bacterial or parasitic infections, these were/are not given much attention and usually the diagnosis is very late. The approach of fungi in developing countries is on gross morphological features while in the developed countries it is the molecular approach that is important. Moreover, life threatening fungal infections is more common in developed countries when compared to developing countries. The incidences of mycotic infections are found to be increasing. The diseases not prevalent in one area are now being reported very frequently due to traveling across the globe. Fungi form a significant cause of morbidity and mortality among the humans and animals. They have emerged as important etiological agents of opportunistic infections as well as full-fledged diseases as true pathogens. It is well known that fungi that are found to be saprophytic, can adapt itself to infect animals and human. Hence there is need to study the life cycle of fungi and their role in biological niche. Only limited data are available about the survival of fungi that commonly cause nosocomial infections in the compromised patients on typical hospital material and this reveals that certain fungi like *Candida, Aspergillus* and *Fusarium* species can survive for atleast for one day or longer on fabrics and plastics routinely used in hospitals and these may act as a reservoirs or vectors for fungi.

**Candida**

Earlier *Candida albicans* was considered as a culture contaminant while analyzing urinary tract and reproductive tract infections; later it was considered as a commensal and then was found to be an opportunistic pathogen inhabiting these regions. However in the span of the last two decades we have witnessed the emergence of this fungus as a major human pathogen.

**Taxonomic position of Candida albicans**: (Alexopolus and Mims, 1983)

Super Kingdom : Eukaryonta
Kingdom : Myceteae  
Division : Amastigomycota  
Sub division : Deuteromycotina  
Form-class : Deuteromycetes  
Form-subclass : Blastomycetidae  
Form-order : Cryptococcales  
Form-family : Cryptococcaceae  
Form-genus : Candida  
Form-species : albicans  

This genus consists of approximately 200 species. (Warren and Hazen, 1999). This number is not immutable due to reassignment of species and discovery of new species by technological advances affecting taxonomic relationships. Since true sexual reproduction is absent in this species it is included in the form class Deuteromycetes. This is a form genus and is heterogeneous in nature. Telomorphs of several genera have been demonstrated for different species of *Candida* and they are *Clavispora*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces* and *Pichia* and thus the genus *Candida* is a mixture of unrelated species belonging to different yeast genera. The main reason for the recognition of a wide variety of species under *Candida* is due to its definition. The genus designation is used for any asexual yeasts which does not have one of the following features:  
(i) acetic acid production; (ii) visually detectable red, pink, or orange pigments; (iii) arthroconidia; (iv) unipolar or bipolar budding on a broad base; (v) blastoconidia formed on sympodulae; (vi) buds formed on stalks; (vii) needle-shaped terminal conidia; (viii) triangular cells; (ix) enteroblastic-basipetal budding usually with mucoid colonies and the ability to grow on inositol as a sole carbon source; and (x) ballistoconidia.  

**Characteristic features of Candida albicans**
The fungus, *Candida albicans* is considered by NNIS (National Nosocomial Infections Surveillance, US) as the **seventh** most prevalent hospital pathogens and **fourth** among urinary tract infection-causing organisms. *Candida albicans* is significant among the 200 so far recognized species of *Candida*.

*Candida albicans* is pleiomorphic and undergoes reversible morphogenetic transitions between budding, pseudohyphal and hyphal growth forms that promote the virulence of this pathogenic fungus. Many view pseudohyphal cells, as an intermediate between the morphological extremes represented by yeast and hyphal cells. Yeast phase (unicellular) is dominant over the pseudomycelial and mycelial (hyphal) forms.

Carbohydrate and lipid metabolism of this species resemble to that of *Saccharomyces cerevisiae*. N-acetyl glucosamine (poor in Carbon and Nitrogen), high temperature (37°C), high CO₂ : O₂ ratio, proline (poor in Nitrogen), starvation all result in germ-tube formation, while low temperature, acidic pH, air and enriched media favor yeast phase. Cell wall proteins and SAP family proteins are generally involved in pathogenecity of *Candida albicans*.

*Candida albicans* is a diploid fungus, which reproduces asexually by means of chlamydospore production (terminal and intercalary) and vegetatively by means of budding (like that of yeast). Sexual reproduction has not been observed in 100 years of research on *Candida* (Perdue, 2000). But recent studies revealed that chances of genetic recombination by sexual reproduction are possible in *Candida* (Tibayrenc, 1997).

*C. albicans* genome size is about 16 Mb (haploid), about 30% greater than *S. cerevisiae* (baker’s yeast) (http://albicansmap.ahc.umn.edu/). It is a diploid fungus with 8 pairs of homologous chromosomes. They are chromosome R, 1, 2, 3, 4, 5, 6 and 7. This numbering of chromosome is based on size of the chromosome from 1 to 7, while chromosome R has variability in size and codes for ribosomal genes. Three ARS elements are noted and they are ARS1 in
chromosome 6, ARS2 in chromosome R and ARS3 in chromosome 7. Apart from this, mitochondrial genome is present; it is circular and 40kb in size. It was concluded that genes coding for integrin like protein, PHR1, PHR2 and SAP & PL and few other genes are involved in pathogenesis. (http://albicansmap.ahc.umn.edu/)

Infections caused by *Candida* are referred as **candidosis or candidiasis**, and both the terms were used in the literature as synonyms. The International Society for Human and Animal Mycology (1980) has suggested the term "candidosis", while the Council for International Organizations of Medical Sciences (1982) recommends "**candidiasis**". The later term will be used in this thesis.

*C. albicans* is the most prominent among the candidiasis-causing *Candida* sps. The question of whether there are subspecies in *C. albicans*, arises since it usually multiplies vegetatively and clones derived are genetically identical. It has possibility for both recombination and clonality.

Based on the area of infection it may be, superficial or systemic candidiasis. Superficial candidiasis includes, oral thrush, oesophageal thrush, cutaneous, vulvovaginal candidiasis and ulcers on cornea of eyes and maceration of finger clefts. Systemic candidiasis includes infection on respiratory, circulatory, urinary and central nervous systems.

This species occurs in soil, plant material, animals, including invertebrates and animal feces. It is isolated from warm-blooded animals, including humans as a part of the normal flora of mucous membranes and not uncommonly, however, it may also become pathogenic, causing **candidiasis**.

*C. albicans* lives as a commensal in oral cavity and vaginal mucosa; generally under the influence of certain predispository factors it becomes a pathogen. These include immuno-compromised state, presence of other diseases, physiological disorders, obesity, alcoholism, use of broad spectrum antibiotics
and steroids all of which contribute to the creation of conditions under which *Candida albicans* becomes pathogenic.

**Antifungal Drugs**

Fungi have ergosterol in the cytoplasmic membrane and it is the most important site of action of many antifungal drugs. Antifungal drugs are classified into the following main groups (Jagdish Chander, 2002).

**A. Antifungal antibiotics**

1. Polyene antibiotics (interferes with sterol synthesis leading to disruption of fungal cell wall)
   
   - Amphotericin B (Conventional & Liposomal formulations)
   - Nystatin
   - Pimaricin
   - Hamycin

2. Other antibiotics
   
   - Griseofulvin (inhibits fungal mitosis by interfering with polymerized microtubules and spindle formation in dividing cells)
   - Pradimicin (acts through calcium-dependent binding to mannans in cell wall)

**B. Synthetic antifungal agents (Chatwal, 1997)**

1. Thiacarbamates (used as topical antifungal agent) e.g. Tolnaftate
2. Allylamines and Benzylamines (inhibits the key enzyme Squalene epoxidase, which is required for ergosterol biosynthesis)
   
   - e.g. Naftifine, Terbinafine, Butenafine
3. Azoles (interact with cytochrome P-450 enzyme systems in fungal cells, resulting in impaired ergosterol biosynthesis)
   
   (i) Imidazoles
   
   - e.g. Bifonazole, Butoconazole, Clotrimazole and Econazole
(ii) Triazoles

  e.g. Fluconazole, Itraconazole, Voriconazole and Teraconazole

C. Miscellaneous antifungal agents

Flucytosine (synthetic fluopyrimide get converted by fungal cytosine deaminase to 5-fluouracil (antimetabolite) which inhibits thymidylate synthetase affecting DNA synthesis)

Ciclopiroxolamine (drug accumulates 200 times more than surrounding medium, inhibits membrane transfer of the aminoacid leucine, at high concentrations, alter cell membrane integrity, leading to leakage of intracellular vital molecules)

Whitfield's ointment (Benzoic acid : Salicylic acid - 2:1, benzoic acid is mild fungistatic agent, while salicylic acid is a keratolytic and a weak antifungal agent)

Potassium iodide (has no demonstrable in vitro antifungal activity and in vivo probably it resolves granuloma, so that body defense mechanism can attack fungus)

Selenium sulfide (it is toxic and used for dandruff)

Echinocandin (Caspofungin) (prevents cell wall synthesis of fungi by blocking β- (1,3)-D-glucan synthase enzyme)

Nikkomycin (inhibits chitin synthetase required for cell wall synthesis)

The antifungal drug resistance can be of two broad categories (Jagdish Chander, 2002)

1. Clinical resistance

  Clinical resistance indicates lack of clinical resistance to the antifungal drugs used in that particular disease.
2. **In vitro** resistance

It is further subdivided into two:

(i) Primary resistance - known as intrinsic or innate resistance and occurs when the organism is naturally resistant to antifungal agents.

(ii) Secondary (acquired) resistance - isolate becomes resistant to the antifungal agent during the course of treatment, which was rare in the past, is now more frequently reported in immunocompromised patients (like AIDS).

Drugs from the polyene class of antifungal agents, specifically amphotericin B, have long been considered the most effective of the systemically administered antifungal agents. The fungicidal antifungal drug **Amphotericin B** has **in vitro** and **in vivo** activity against yeasts (**Candida** spp., **Cryptococcus neoformans**), molds (**Aspergillus** spp., **Zygomycetes**, dematiaceous fungi), and dimorphic fungi. Unfortunately, infusion-related toxicities, the frequent association of renal dysfunction, and the intravenous formulation of amphotericin B have limited the utility of this drug.

Allylamine resistance has not been reported for medically important fungi, although resistant strains have been described in **S. cerevisiae** and the plant pathogen **Ustilago maydis** (Vanden Bossche *et al.*, 1994). Resistance to morpholines or thiocarbamates has not been reported. However, as with any antimicrobial agent, prolonged use will probably select for strain replacement with a resistant isolate or the development of secondary resistance in the original sensitive isolate.

Similarly, **5-Flucytosine**, although generally active against **Candida** spp., **C. neoformans**, and some molds, has limited clinical utility owing to the frequent association of hematological toxicity and the rapid development of resistance, particularly when it is used as a single agent (Bennett, 1996.). Primary or
intrinsic resistance to this drug is a common phenomenon. Estimates suggest that 10% of *C. albicans* clinical isolates are intrinsically resistant and that 30% will develop secondary resistance (Vanden Bossche *et al.*, 1994). The genetics of 5-FC resistance has been investigated in several fungi.

The azole antifungal agents, because of their relative safety and ease of delivery, have subsequently become a critical component in the antifungal armamentarium.

**Need for new anti-fungal drugs**

The rise in the incidence fungal infections has exacerbated the need for the next generation of antifungal agents, since many of the currently available drugs have undesirable side effects, are ineffective against new or reemerging fungi, or lead to the rapid development of resistance as indicated above (White *et al.*, 1998). New classes of antifungal drugs, that too of plant origin i.e., not synthetic will clearly be important for future treatment strategies.

Drug discovery (Mitscher, 2004) started from prehistoric period with the use of higher plants and animals and this continues to till date providing biologically active compounds of unanticipated structural types. Adding to the long list of classical plant products that have survived into modern medicine, are substances of more recent origin that include antibiotics (penicillin, cephalosporins, etc.), anticancer agents (taxol, Vinca alkaloids), immunosuppressant drugs (cyclosporins and tacrolimus), pharmacological agents (compactin, asperlicin, etc.), Natural products provide structural patterns for various valuable medications (snake venom led to orally active angiotensin converting enzyme inhibitors, cocaine led to local anesthetics, willow bark glycosides led to aspirin, etc.). A great deal of ethnomedicines that exist are to be mined and so there is a need for hasty bioprospecting.

Plants are very vital in health care. 80% of people in less developed/developing countries still rely only on traditional medicines obtained
from local plants (Krishnamurthy, 2003). 85% of traditional medicines involve the use of plant extracts and by now over 200 chemicals have been extracted in pure form plant species throughout the world. At present only a very small percentage of world’s plants contributes on a global scale to health care. Of the 21,000 medicinal plant species listed by WHO, more than 2,500 species are mentioned in Siddha, Ayurveda, Unani and other traditional health care systems of India.

The general aim of the conventional systems of medicine is to help people maintain health and treating the disease is only secondary. Diseases are viewed from patient’s body point of view, i.e. by giving medicines, that can change the nature of pathogen’s microenvironment (host) and by this infection is arrested and pathogen cannot survive or withstand the change. Unlike Allopathic systems there may not be any harmful side-effects on the host system. There are several medicinally important plants used for promotive, preventive and curative aspects of health. Plants are used as appetisers, wound-dressers, wound-heelers, purgatives, emetics, antidotes for bites of dog, rat, poisonous snakes, insects, etc. and for treatment of tumours, cancers, bone-fractures, diabetes, heart attack, renal failure, helminthic and other worm infestations; they are also used to cure psychological disorders like depression, tension, somnambulism, etc. (Sinha, 1996). They are also used in treatment of bacterial, viral and fungal diseases. The range of plants used in traditional medicinal preparation is so vast and diverse that one may rightly wonder if there is such a thing as a “non-medicinal” plant.

Bioprospecting is the exploration for every valuable genetic and / or biochemical resource that finds use in pharmaceutical, biotechnological and agricultural industries either through bioprocesses unique to them or through novel end products. There are three different methods for bioprospecting: (Krishnamurthy, 2003):
Random – random collection of plants for analysis of their economical potential but the rate of success is very less, tedious and time consuming

Phylogenetic – collection and analysis of members of those families in which some taxa are already known to be sources of some useful products but rate of success is comparatively more and less time consuming than the random method and

Ethnodirected – focuses on certain plants which are reported to have particular properties by tribal people / indigenous community (traditional knowledge) but not yet popularized. It has many advantages than the other two methods. Ethnodirected method is the best because it provides Ready-made knowledge, tested through years of experience, sure to yield desired results, less time-consuming, and research and development costs are significantly reduced

Antifungal susceptibility testing (in vitro studies)

Antifungal susceptibility testing remains an area of intense interest. Susceptibility testing can be used for drug discovery and epidemiology. Although antifungal susceptibility testing remains less well developed and utilized than antibacterial testing, the scientific support for its validity has been benefited greatly by extrapolation from antibacterial testing (Rex et al., 2001). Knowledge of mechanisms of antifungal resistance has been valuable in identifying resistant isolates and using them to validate in in vitro measurement systems (Johnston and Siegel, 1990 and Radhika et al., 1989).

Use of animals in scientific investigation i.e. preclinical trials (Fort, 2002) can be traced back to several centuries BC. Earlier, these experiments were carried out using domestic or easily captured species but by the end of nineteenth century, the concept of laboratory animals began to emerge and animals are bred in captivity or from its environment for its usefulness in specific investigations at hand. Two to three decades ago the initial screening of new compounds for pharmacological activity was conducted using whole animals and organs or
tissues isolated from animals. But now initial screening is done in *in vitro* conditions (outside the living system) and only after identification of new drug candidate, animal studies are initiated. New drugs must be tested *in vivo* (pre-clinical trials) before going in for clinical trials in human beings. The effects of the processes of absorption, distribution, metabolism, excretion and interactions among these processes and interactions among the various organs and neuroendocrine systems within the whole animal cannot be duplicated in *in vitro*. Moreover to study effectiveness of the drug inside a living system, effective concentration and safety doses, are to be decided; besides these one must study the adverse effects and side effects and possible allergic reactions in animal models are used. Proper care of laboratory animals used in research is a basic requirement to assure the validity and reproducibility of the results obtained.

The major therapeutic classes of drugs that generally need animal studies are:

(i) CNS (sedative, hypnotic, anesthetic, analgesic, etc.)
(ii) Cardiovascular (Anti-hypertensive, anti-anginal, anti-thrombic, cardiotonic, etc.)
(iii) Metabolic (hypolipidemic, diuretic, etc.)
(iv) Immunopharmacologic (anti-inflammatory, anti-edema, anti-allergic, etc.)
(v) Gastrointestinal (anti-ulcer, anti-secretory, etc.)
(vi) Antimicrobial (anti-bacterial, anti-fungal and anti-viral)

**Clinical trials**

A **clinical trial** (also clinical research) is a research study in human volunteers to answer specific health questions. ([http://www.clinicaltrials.gov/ct/info/resources](http://www.clinicaltrials.gov/ct/info/resources)).

A protocol is a study plan on which all clinical trials are based. The plan is designed to safeguard health of participants as well as answer specific research questions. It describes what type of people may participate in the trial; the
schedule of tests, procedures, medications, and dosages; and the length of the study. Participants following the protocol should be monitored for health conditions, and safety and effectiveness of their treatment should be determined.

A placebo is an inactive pill, liquid or powder that has no treatment value. It is generally used in comparison with treatments to assess the treatment's effectiveness. In some studies participants in the control group will receive placebo instead of an active drug or treatment.

A control is the standard by which experimental observations are evaluated. In clinical trials mostly, one group will be given the experimental drug while the control group will be given either a standard treatment or placebo.

Clinical drug development is generally divided into following four types:

**Treatment trial**  test new treatments, new combinations of drugs, or new approaches to surgery or radiation therapy

**Prevention trial**  look for better ways to prevent disease or to prevent a disease from returning. These may include medicines, vitamins, vaccines, change of life style, etc.

**Screening trial**  test the best way to detect certain diseases or health conditions

**Quality of Life trial**  explore ways to improve comfort and quality of life for individuals with chronic illness

Clinical trials are conducted in phases and each phase has a different purpose and helps scientists answer different questions:

In **Phase I trials**, researchers test a new drug or treatment in a small group of people (20-80) for the first time to evaluate its safety, determine a safe dosage range, and identify side effects.

In **Phase II trials**, the study drug or treatment is given to a larger group of people (100-300) to see if it is effective and to further evaluate its safety.
In Phase III trials, the study drug or treatment is given to large groups of people (1,000-3,000) to confirm its effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow the drug or treatment to be used safely.

In Phase IV trials, post marketing studies delineate additional information including the drug’s risks, benefits and optimal use.

Clinical research represents a vital stage in the development process a stage that is no less challenging than the preclinical research stage. (Cato et al., 2002). For every clinical study input comes from multiple personnel with various areas of expertise, which include physicians, scientists, pharmacists, project managers, statisticians, computer programmers, study monitors, regulatory experts, and for some studies, a representative of the formulations group with these team it would take at least 13 to 20 years time from drug discovery to marketing. Scientists (Ph.D.s) are trained primarily in basic research while physicians (MDs) are trained in clinical medicine and a single drug development program is derived from both of these distinct disciplines, considerable overlap, cooperation and coordination are necessary to take a drug successfully and efficiently from discovery to market.

Need for the Present study

A number of plants have been listed as having anti-candidal activity in traditional medical systems of India, but not much work has been directed to exploit them and to evaluate their potential. Hence, this work has been undertaken.

The main objectives of this work are listed below:

1. Selection of plants referred to in the Siddha, Ayurveda, Unani and other traditional systems (ethnodirected method) of medicine in India for the antifungal activity especially anti-candida activity.
2. Selection of a particular *Candida albicans* isolate among those made available and a MTCC (227) strain for antifungal study and standardization of conditions for maintaining them.

3. Standardization of *in-vitro* antifungal assay by agar disc diffusion assay and choosing the plant extract of specific solvent and concentration that shows inhibition of fungal growth.

4. Phytochemical analysis of effective extracts.

5. Formulation of a drug

6. Evaluation of its activity in animal model, and

7. Effectiveness of the drug in human beings